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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	HYG 1194-011D
First Inventor	Wille, Jr.
Title	PROTEIN-FREE DEFINED MEDIA FOR THE GROWTH OF NORMAL HUMAN KERATINOCYTES
Express Mail Label No.	EL039917014US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: **Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231**

1. [X] **Fee Transmittal Form (e.g., PTO/SB/17)** *(Submit an original and a duplicate for fee processing)*
2. [X] **Applicant claims small entity status.** See 37 CFR 1.27.
3. [X] **Specification** [Total Pages 36] *(preferred arrangement set forth below)*
   - Descriptive title of the invention
   - Cross Reference to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to sequence listing, a table, or a computer program listing appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings (if filed)
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
4. [X] Drawing(s) (35 U.S.C. 113) [Total Sheets 11]
5. Oath or Declaration [Total Pages 3]
   a. [ ] Newly executed (original or copy)
   b. [ ] Copy from a prior application (37 CFR 1.63 (d)) *(for continuation/divisional with Box 17 completed)*
      i. [ ] DELETION OF INVENTOR(S) **Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).**
6. [ ] Application Data Sheet. See 37 CFR 1.76
7. [ ] **CD-ROM or CD-R in duplicate, large table or Computer Program** *(Appendix)*
8. **Nucleotide and/or Amino Acid Sequence Submission** *(if applicable, all necessary)*
   a. [ ] **Computer Readable Form (CRF)**
   b. **Specification** Sequence **Listing on:**
      i. [ ] **CD-ROM or CD-R (2 copies); or**
      ii. [ ] **paper**
   c [ ] Statements verifying **identity of above copies**

ACCOMPANYING APPLICATION PARTS

9. [ ] Assignment Papers (cover sheet & document(s))
10. [ ] 37 CFR 3.73(b) Statement *(when there is an assignee)* [ ] Power of Attorney
11. [ ] English Translation Document *(if applicable)*
12. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
13. [ ] Preliminary Amendment
14. [X] Return Receipt Postcard (MPEP 503) *(Should be specifically itemized)*
15. [ ] Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
16. [ ] Other: check

17. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in a preliminary amendment, or in an Application Data Sheet under 37 CFR 1.76:*

[ ] Continuation [ ] Divisional [X] Continuation-in-part (CIP) of prior application No. 09 /271,777

Prior application information: Examiner L. Lankford, Jr. Group/ Art Unit: 1651

For CONTINUATION OR DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 6b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

18 CORRESPONDENCE ADDRESS

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Name (Print/Type)	Carol G. Stovsky	*Registration No. (Attorney/Agent)*	42,171
Signature	Carol G. Stovsky	*Date*	10-23-2000

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FEE TRANSMITTAL for FY 2000

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT ($) 404.00

Complete if Known	
Application Number	
Filing Date	October 23, 2000
First Named Inventor	Wille, Jr.
Examiner Name	
Group Art Unit	
Attorney Docket No.	HYG 1194-011D

METHOD OF PAYMENT (check one)

1. ☐ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

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Deposit Account Name

☐ Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17

☐ Applicant claims small entity status See 37 CFR 1.27

2. ☒ Payment Enclosed:

☒ Check ☐ Credit card ☐ Money Order ☐ Other

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	710	201	355	Utility filing fee	365.00
106	310	206	155	Design filing fee	
107	480	207	240	Plant filing fee	
108	690	208	345	Reissue filing fee	
114	150	214	75	Provisional filing fee	

SUBTOTAL (1) ($) 365.00

2. EXTRA CLAIM FEES

			Extra Claims		Fee from below		Fee Paid
Total Claims	6	-20 ** =	0	x		=	
Independent Claims	4	-3 ** =	1	x		=	39.00
Multiple Dependent						=	39.00

** or number previously paid, if greater, For Reissues, see below

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	78	202	39	Independent claims in excess of 3
104	260	204	130	Multiple dependent claims, if not paid
109	78	209	39	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 39.00

FEE CALCULATION (continued)

3.ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2520	147	2520	For filing a request for ex parte reexamination	
112	920	112	920	Requesting publication of SIR prior to Examiner Action	
113	1840	113	1840	Requesting publication of SIR after Examiner Action	
115	110	215	55	Extension for reply within first month	
116	380	216	190	Extension for reply within second month	
117	870	217	435	Extension for reply within third month	
118	1360	218	680	Extension for reply within fourth month	
128	1850	228	925	Extension for reply within fifth month	
119	300	219	150	Notice of Appeal	
120	300	220	150	Filing a brief in support of an appeal	
121	260	221	130	Request for oral hearing	
138	1510	138	1510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1210	241	605	Petition to revive - unintentional	
142	1210	242	605	Utility issue fee (or reissue)	
143	430	243	215	Design issue fee	
144	580	244	290	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt.	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	690	246	345	Filing a submission after final rejection (37 CFR Section 1.129(a))	
149	690	249	345	For each additional invention to be examined (37 CFR Section 1.129(b))	
179	710	279	355	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify):

* Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($)

SUBMITTED BY

Complete *(if applicable)*

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Signature	Carol G. Stovsky			Date	10-23-2000

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Express Mail No.: EL039917014US Date: October 23, 2000

APPLICATION FOR UNITED STATES LETTERS PATENT

FOR

PROTEIN-FREE DEFINED MEDIA FOR THE GROWTH OF NORMAL HUMAN KERATINOCYTES

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PROTEIN-FREE DEFINED MEDIA FOR THE GROWTH OF NORMAL HUMAN KERATINOCYTES

Inventor: John J. Wille, Jr.

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Serial No. 09/271,777, now U.S. Patent No. _____ issued _____, entitled _____; which is a continuation of U.S. Serial No. 09/133,386, filed August 13, 1998, now U.S. Patent No. 5,912,175; which is a continuation of U.S. Serial No. 08/893,195, filed July 15, 1997, now U.S. Patent 5,834,312 issued November 10, 1998, entitled PROCESS AND MEDIA FOR THE GROWTH OF HUMAN EPITHELIA; which is a continuation-in-part of U.S. Serial No. 08/500,744, filed July 11, 1995, now U.S. Patent 5,686,307 issued November 11, 1997, entitled SERUM-FREE MEDIUM FOR USE IN THE FORMATION OF A HISTOLOGICALLY COMPLETE LIVING HUMAN SKIN SUBSTITUTE; which is a continuation of U.S. Serial No. 08/318,221, filed October 5, 1994, now abandoned; which is a continuation of U.S. Serial No. 08/184,905, filed January 21, 1994, now abandoned; which is a continuation of Serial No. 08/063,247, filed May 18, 1993, now abandoned; which is a divisional of U.S. Serial No. 07/471,976, filed January 29, 1990, now U.S. Patent No. 5,292,655. U.S. Patents 5,292,655, 5,686,307, 5,834,312, 5,912,175, and _____ are incorporated herein by reference.

TECHNICAL FIELD OF THE INVENTION

The field of the invention is in biology and more specifically in the subspecialty of cell biology. The invention relates to a process and cell culture media for the growth

of human epithelia, such as gingival epithelium, ureteral epithelium, and corneal epithelium.

BACKGROUND OF THE INVENTION

The epithelium is the membranous cellular tissue that covers the surface or lines a tube or cavity of an animal body. The epithelium serves to enclose and protect the other parts of the body and may produce secretions and excretions and may be associated with assimilation as seen in the gastrointestinal tract. The epithelium is one of the four primary tissues of the body, which constitutes the epidermis and the lining of respiratory, digestive and genitourinary passages. The cornea, which is the transparent part of the coat of the eyeball that covers the iris and pupil and admits light to the interior, is also a tissue that is made of epithelial cells.

The functions of epithelia are varied and include: (1) protective function, by completely covering the external surface (including the gastrointestinal surface, the surface of the whole pulmonary tree including the alveoli and the eye); (2) secretory function, by secreting fluids and chemical substances necessary for digestion, lubrication, protection, excretion of waste products, reproduction and the regulation of metabolic processes of the body; (3) absorptive function, by absorbing nutritive substances and preserving water and salts of the body; (4) sensory function, by constituting important parts of sense organs, especially of smell and taste; and (5) lubricating function, by lining all of the internal cavities of the body, including the peritoneum, pleura, pericardium and the tuncia vaginalis of the testis.

The growth of human epithelial cells without the use of companion-cells, protein growth factors, feeder layers, serum components or organotypic substrates is the advancement in the state of the art that is this invention. Traditionally, tissue culture of normal epithelial cells has been attempted in a variety of commercially available media designed for the growth of less fastidious types of cells, i.e., malignant cells transformed in vitro from cell lines derived from human or non-human tissues, cell lines developed from human or non-human tumors, or cell lines developed for human or non-human embryonic mesenchymal cell types. In contrast, the culture of normal human epithelial stem cells has presented many difficulties not the least of which is the inexorable tendency for these cells to undergo uncontrolled, irreversible, terminal differentiation with the consequent loss of cell division capacity.

A significant development made by Tsao et al. is the formulation of a nutrient medium supplemented with specified growth factors and hormones allowed for the growth of human epidermal cells. See Tsao, M. C. et al., J Cellular Physiol. 110:219-229 (1982). The Tsao medium has been designated MCDB 152. Further refinements of this medium lead to the development of a medium known as MCDB 153. See Boyce, S. T. and Ham, R. G., J Invest. Dermatol. 81:33-40 (1983). The use of these media permitted a more accurate characterization of the necessary growth factors, hormones and Ca^{2+} requirements for retention of high cloning efficiency which is necessary to maintain proper genetic programming for continued subculture of pluripotent basal epidermal stem cells. See Wille, J. J. et al., J Cellular Physiol. 121:31-44 (1984).

The use of serum in cell culture medium provides a complex mixture of growth factors and differentiation-inducing factors. See Pittelkow, M. R. et al., J Invest,

Dermatol. 86:410-417 (1986). Pittelkow et al. reported that serum, known to contain fibroblastic cell growth factors, e.g., platelet-derived growth factor, was an inhibitor of basal epidermal cell growth. Further, the differentiation-inducing factors in serum could be equated with serum's content of β-transforming growth factor, (β-TGF). See Shipley, S. D. et al., Cancer Res. 46:2068-2071 (1986). It has also been reported that normal human keratinocytes actually produce their own growth factors. That is, proliferating basal cells are stimulated to secrete α-transforming growth factor (α-TGF) in the presence of added epidermal growth factor (EGF) and decrease production of α-TGF at high cell densities near confluence. Under the latter condition, the arrested cells secrete an inactive form of β-TGF. See Coffey, R. J. et al., Nature 328:817-820 (1987). These considerations led the inventor to the idea that the natural mechanism of growth stimulation and its regulation in cultured epithelia cells could be accomplished through manipulation of the various media components and that such manipulation would also eliminate the need for an organic substrate or organotypic matrix as well.

Judd et al. discuss a keratinocyte growth medium designated keratinocytes-SFM in an article entitled: "Culture of Human Keratinocytes in Defined Serum Free Medium", Focus, 19, No. 1, Pgs. 1-5. This serum-free media is also disclosed in a Gibco Product brochure. However, the actual composition of the SFM media is not disclosed other than it does not contain the growth promoting additives insulin, epidermal growth factor and fibroblast growth factor.

An article by Wille et al., in J Dental Research, 68:1019 (1989) entitled "Serum Free Cultures of Normal Human Gingival Keratinocytes (HGK)" discusses the successful in vitro culturing of human gingival keratinocytes in MCDB 153 medium, supplemented

with 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 0.5 mM hydrocortisone, 5 μg per ml epidermal growth factor, 5 μg per ml insulin and 35 μg per ml bovine pituitary extract protein where the presence of these proteins is necessary, but their function is unknown in this heterogeneous tissue extract mixture. Wille et al. in The Journal of Cellular Physiology, 150:52-58 (1992) in an article entitled "Effects of Growth Factors, Hormones, Bacterial Lipopolysaccharides and Lipotechoic Acids on the Clonal Growth of Urethreal Epithelial Cells in Serum Free Culture", discloses the use of F-12 media containing bovine pituitary extract and bovine serum albumin for culturing cells isolated from human ureters, again where such tissue products have necessary but unknown effective components.

Chopra et al. in the Journal of Cellular Physiology, 130:173-181 (1987) entitled: "Propagation of Differentiating Normal Human Tracheobronchial Epithelial in Serum Free Medium" discloses the use of a medium similar to MCDB 151 except that it contains 5.4 μg per ml HEPES, 6.1 μg per ml sodium chloride, 0.3 μg per ml sodium acetate and 1 μg per ml sodium bicarbonate. These changes lowered the final osmolarity of the disclosed medium to 290 mosmols. The concentration of HEPES in the Chopra et al. solution was 28 mM.

U.S. Pat. No. 5,328,844 to Moore discloses a culture medium useful for establishing, growing and maintaining mammalian cells in culture, in particular for the establishment of culture of human, normal and malignant cells. The claimed media contains 4,500 mg per liter of HEPES and 5 mg per liter of insulin. This patent does not relate to nor disclose media useful for growth of normal epithelial cells.

In an article by Boisseau et al. entitled "Production of Epidermal Sheets in a Serum Free Culture Medium: A Further Appraisal of the Role of Extracellular Calcium", Journal of Dermatological Science, 3 (1992), 111-120, the author discloses the serum-free media (MCDB 153) to grow keratinocyte monolayers in clonogenic conditions. The effect of extracellular calcium and temperature on proliferation and differentiation of cultured keratinocytes was investigated.

U.S. Pat. No. 4,673,649 to Boyce et al. discloses a basal medium which was MCDB 152 supplemented with epidermal growth factor, transferin, insulin, hydrocortisone, ethanolamine, phosphoethanolamine and progesterone to obtain a medium for growth of human keratinocytes. The inventor of the present application in U.S. Pat. No. 5,292,655 demonstrates that progesterone inhibits optimal growth.

Wilke et al. in "Biologic Mechanisms for the Regulation of Normal Human Keratinocyte Proliferation and Differentiation", American Journal of Pathology, Vol. 131, No. 1, April, 1988, describe a serum-free medium with low calcium concentrations on the order of 0.1 mM. These studies of Wilke et al. actually used MCDB 153 medium supplemented with insulin, EGF and protein of bovine pituitary extract where any effective components are unknown in the extract.

U.S. Pat. No. 5,232,848 to Wolfe et al. discloses a nutrient medium for both high and low density culture of a wide variety of non-epithelial cell lines and cell types. This patent discloses and claims a zwitterionic buffer such as HEPES at a concentration of 2.5 x 10^{-2} moles.

Boyce et al. in U.S. Pat. No. 4,940,666 discloses and claims a growth medium which is free of transferin, comprising complete MCDB 153, EGF and insulin.

Nissley et al. in "Growth and Differentiation of Cells in a Defined Environment", pgs. 337-344 discloses that cells of embryonic and fetal origin produce IGF-1 and IGF-2 which may be important for the control of embryonic and fetal growth. The authors also suggest that the use of these cells could potentially stimulate the growth of the same or neighboring cells and thereby avoid the inclusion of such growth factors in a culture medium.

Boyce et al. in "Calcium Regulated Differentiation of Normal Human Epidermal Keratinocytes in Chemically Defined Clonal Culture and Serum Free Serial Culture", in The Journal of Investigative Dermatology, 81:33S-40S (1983) discloses MCDB 153 supplemented with a number of growth factors and an optimum level of calcium at 0.3 mM for colony forming efficiency and a high calcium concentration of 1.0 mM for induction of stratification and terminal differentiation.

U.S. Pat. No. 5,326,699 to Torishima et al. discloses a serum-free medium for culturing animal epithelial cells comprising 8-14 μg per ml (53.6 mM -93.8 mM) of methionine, up to 0.1 mM of calcium in the form of calcium chloride and other conventional ingredients such as glucose, growth factors, buffers and the like.

U.S. Pat. No. 5,686,307 and U.S. Pat. No. 5,834,312 to Wille disclose a serum-free medium for culturing animal epithelial cells comprising the amino acid histidine (3.0-33 mg/L), isoleucine (3.0-33 mg/L), methionine (4.5-45 mg/L), tryptophan (4.0-44 mg/L) and tryosine (5.0-55 mg/L), NaCl (90-140 mM) and Hepes (14-22 mM) that is useful for the production of a living human skin and animal epithelia. This medium (HECK 109) requires EGF and IGF-1 as the only two protein growth for serial cultivation of proliferating cultures of normal human keratinocytes.

Varani et al. have reported that all-trans retinoic acid stimulates the growth of adult human keratinocytes cultured in a growth factor-deficient medium. Early passage keratinocytes were incubated for 1 or 2 days in a serum-free keratinocyte growth medium (MCDB 153) supplemented with EGF, insulin and BPE and 1.4 mM Ca^{2+} or in growth factor-deprived keratinocyte basal MCDB 153 medium. The cells were concomitantly treated with all-trans retinoic acid (0.1 – 2.5 ng/ml). Treatment with all-trans retinoic acid inhibited proliferation of keratinocytes that were rapidly growing in the growth-factor supplemented medium. By contrast, all-trans retinoic acid treatment of keratinocytes in growth-factor deficient medium, in which the cells were growth arrested, stimulated growth. Stimulation was observed in a serum-free medium lacking not only protein growth factors, but hydrocortisone, ethanolamine, and phosphoethanolamine. The rate of keratinocyte proliferation in the retinoid-stimulated cultures was approximately 35% of the maximal proliferation rate observed in growth factor supplemented medium. It should be noted that the optimal concentration of all-trans retinoic acid required to produce these effects was 0.5 ng/ml (1.6×10^{-6}M). This is about 100-fold greater than the physiological concentration, and is present in amounts known to be damaging to cell membranes. Lower concentration of all-trans retinoic acid were ineffective.

In addition, Marcelo and Dunman (1997) reported that retinoic acid stimulates essential fatty acid-supplemented human keratinocytes. These results were observed in keratinocyte cultures grown in a serum-free medium (MCDB 153) that was supplemented with the protein growth factors, EGF, insulin, and BPE. Finally, Kamata et al. (1999) has reported the growth of oral keratinocytes in a novel protein-free defined medium call

PF86-a (Rikimaru et al., 1990) with 85% serum-free medium, MCDB 153 (U.S. Pat. No. 4,673,649). No explanation or hypothesis was given as to what element(s) of the composition were responsible for the ability of these medium to support keratinocyte growth in the absence of protein growth.

A medium that eliminates the use of growth factors in a defined medium would have many technical and commercial benefits. In order to accomplish this goal, the inventor has replaced EGF and IGF-1 in a novel serum-free medium with retinyl acetate. Studies show that sustained growth of human keratinocytes is readily achieved in this improved serum-free medium. In addition, retinyl acetate stimulates proliferation at physiological concentrations unlike the reported effect of all-trans retinoic acid (Varani et al., 1989) and in HECK 109 serum-free medium supplemented with growth hormones (hydrocortisone, ethanolamine, and phosphoethanolamine).

Pellegrini in "Long Term Restoration of Damaged Corneal Surfaces with Autologous Cultured Corneal Epithelium", Lancet, Vol. 349 (1997) discloses the culturing of corneal cells in Dulbecco, Vogt, Eagle's and Ham's F-12 Media containing fetal bovine serum, insulin, transferin, EGF and cholera toxin. The authors' report that cells isolated from the central cornea (limbus) and bulbar conjunctiva could be grown in vitro and then transplanted to the human host.

U.S. Pat. No. 4,304, 866 to Green et al. discloses an in vitro method for the formation of epithelial sheets from cultured keratinocytes. The Green method uses a serum containing medium and a feeder layer of murine (mouse) fibroblast cells to accomplish cell growth and differentiation. This procedure has serious limitations for large scale production of human epithelium as the use of serum inextricably confounds

the culture of purely basal cells with the dynamics of serum-induced differentiation. The net result is that sub-cultivation of such cultures yields low (<5%) clonal efficiencies preventing step wise large scale build up of uncommitted pluripotent basal cells as a prelude to their conversion into usable sheets of transplantable, histologically-complete, human epithelium. Moreover, the process of Green et al. does not describe the formation of a histologically complete epidermis. The Green et al. procedure forms an epidermis lacking a stratum corneum which is necessary for maximizing the utility of the tissue.

Prior art methods have achieved a complete epidermis, but only in the presence of a complete skin starter sample and serum-containing media that are combined with an organotypic substratum containing growth factors produced by companion cells as disclosed in U.S. Pat. No. 4,485,096. The use of any organotypic substrate as well as feeder or companion cell types, e.g. fibroblasts, seriously limits the resulting products safety and economic viability. See Nanchahal, J. et al. in Lancet II(8656):191-193, (1989).

In order to remedy these deficiencies, the inventor has dispensed with serum-containing media, eliminated any substratum support, dispensed with the requirement for innumerable skin starter samples, and designed a novel and unobvious medium capable of supporting the growth and development of a complete epithelium. Moreover, the identification of essential process steps leading to a functional epithelium has been discovered and can be monitored with specific monoclonal antibodies. The prior art media which contain undefined serum and/or feeder cell factors and/or organotypic substrates and millimolar concentrations of Ca^{2+}, high levels of buffers, inadequate levels of amino acids and incorrect osmolalities were not designed for the unlimited

proliferation of undifferentiated basal cells. The prior art media allows cultures to spontaneously undergo maturation and uncontrolled differentiation. In contrast, the serum-free media described in this invention produces a complete epithelium.

SUMMARY OF THE INVENTION

There is disclosed an aqueous solution for isolating epithelial cells from animal tissue, said solution comprising:

a) glucose at a concentration of about 10 mM;

b) N-(2-OH-ethyl-)piperazine-N'-(2-ethanesulfonic acid) (HEPES) at a concentration of 16-22 mM;

c) sodium chloride at a concentration of 90-140 mM;

d) potassium chloride at a concentration of about 3 mM;

e) sodium orthophosphate ($Na_2HPO_4.7H_2O$) at a concentration of 1 mM;

f) phenol red at a concentration of 0.0033 mM;

g) about 100 units of penicillin per ml of solution;

h) about 100 units of streptomycin per ml of solution; and

i) one component selected from the group consisting of:

(i) trypsin at a concentration of 0.1%-0.2% w/v; and

(ii) soy bean trypsin inhibitor at a concentration of 0.1-1.0% w/v.

The aqueous solution for the isolation of the basal epithelial cells or cell competency solution (CCS) is actually two solutions. The first solution contains trypsin to digest the cells of interest from other cellular tissue. The second solution contains a soybean trypsin inhibitor to stop the digestion of the tissue.

In a more preferred embodiment of the cell competency solution, the sodium chloride is at a concentration of 100 to 130 mM; the HEPES is at a concentration of 18 to 21 mM; the trypsin is at a concentration of 0.12 to 0. 18% w/v in the digestion solution and the soybean trypsin inhibitor is at a concentration of 0.3 to 0.8% w/v in the second CCS.

The present invention also relates to a method for the isolation of basal epithelial cells from animal tissues, said method comprising the steps of:

a) obtaining animal epithelium;

b) comminuting said epithelium;

c) placing said comminuted epithelium in the cell competency solution described above containing trypsin at a temperature and for a time sufficient to allow separation of the basal epithelial cells from the epithelium;

d) collecting said epithelial cells; and

e) passaging said basal epithelial cells to the CCS containing soybean trypsin inhibitor.

It should be understood that the method described above uses two (2) CCS solutions: (1) a solution containing trypsin to digest the tissue; and (2) a solution containing a soy bean trypsin inhibitor to terminate the digestion of the tissue.

In its broadest sense, the present invention relates to the use of a serum-free medium for culturing animal epithelial cells comprising:

a) N-(2-OH-ethyl-)piperazine-N'-(2-ethane-sulfonic acid) at a concentration of 14-22 mM;

b) sodium chloride at a concentration of 100-120 mM;

c) histidine at a concentration of 0.1-0.25 mM;

d) isoleucine at a concentration of 0.05-0.5 mM;

e) methionine at a concentration of 0.1-0.5 mM;

f) phenylalanine at a concentration of 0.1-0.5 mM;

g) tryptophan at a concentration of 0.05-0.5 mM; and

h) tyrosine at a concentration of 0.1-0.5 mM.

This serum-free medium of the invention is sometimes hereinafter referred to as the BASAL medium.

In addition, there is disclosed a protein-free defined medium for the culturing of normal human keratinocytes comprising:

a) N-(2-OH-ethyl-)piperazine-N'-(2-ethane-sulfonic acid) at a concentration of 14-22 mM;

b) sodium chloride at a concentration of 100-120 mM;

c) calcium^{2+} ions at a concentration of 0.7-3.0 mM;

d) histidine at a concentration of 0.1-0.25 mM;

e) isoleucine at a concentration of 0.05-0.5 mM;

f) methionine at a concentration of 0.1-0.5 mM;

g) phenylalanine at a concentration of 0.1-0.5 mM;

h) tryptophan at a concentration of 0.05-0.5 mM;

i) tyrosine at a concentration of 0.1-0.5 mM; and

j) retinyl acetate at a concentration of 0.3 ng/ml – 33 ng/ml.

This inventive protein-free defined medium is sometimes hereinafter referred to as HECK-110.

In addition, there is disclosed a serum-free medium for culturing epidermal keratinocytes comprising:

a) N-(2-OH-ethyl-)piperazine-N'-(2-ethane-sulfonic acid) at a concentration of 14-22 mM;

b) sodium chloride at a concentration of 100-120 mM;

c) calcium^{2+} ions at a concentration of 0.7-3.0 mM;

d) histidine at a concentration of 0.1-0.25 mM;

e) isoleucine at a concentration of 0.05-0.5 mM;

f) methionine at a concentration of 0.1-0.5 mM;

g) phenylalanine at a concentration of 0.1-0.5 mM;

h) tryptophan at a concentration of 0.05-0.5 mM;

i) tyrosine at a concentration of 0.1-0.5 mM;

j) β-transforming growth factor at a concentration of 3.0-30 ng/ml; and

k) retinyl acetate at a concentration of 0.3-33 ng/ml.

This inventive serum-free medium is sometimes hereinafter referred to as HECK-110 DM.

There is further disclosed a serum-free medium for culturing epidermal keratinocytes comprising:

a) N-(2-OH-ethyl-)piperazine-N'-(2-ethane-sulfonic acid) at a concentration of 14-22 mM;

b) sodium chloride at a concentration of 100-120 mM;

c) calcium^{2+} ions at a concentration of 0.7-3.0 mM;

d) histidine at a concentration of 0.1-0.25 mM;

e) isoleucine at a concentration of 0.05-0.5 mM;

f) methionine at a concentration of 0.1-0.5 mM;

g) phenylalanine at a concentration of 0.1-0.5 mM;

h) tryptophan at a concentration of 0.05-0.5 mM;

i) tyrosine at a concentration of 0.1-0.5 mM;

j) linoleic acid at a concentration of 1-15 ng/ml; and

k) retinyl acetate at a concentration of 0.3-33 ng/ml.

The above described serum-free medium according to the invention is sometimes hereinafter referred to as HECK-110 CM.

The present invention also relates to a method for the formation of a histologically complete, stratified animal epithelium using the media described herein. More specifically, there is disclosed a method for the formation of a histologically complete, stratified human epithelium comprising the steps of:

a) isolation of basal stem cells from animal epithelium using the CCS that contains trypsin;

b) recovering said basal stem cells using CCS that contains soy bean trypsin inhibitor;

c) culturing said isolated basal stem cells in HECK-110 medium to form a confluent sheet of undifferentiated epithelial tissue;

d) culturing said sheet of undifferentiated epithelial tissue in HECK-110 DM to form a sheet of differentiated and stratified tissue; and

e) culturing said differentiated and stratified tissue in HECK-110 CM to form a cornified epithelium.

In a preferred embodiment, the method of the present invention forms a histologically complete human skin.

The invention further relates to the formation of a differentiated and stratified tissue wherein the method is set forth above with the omission of step e) wherein the cornified epithelium layer is formed. The inventive method wherein cornification is omitted is preferably applied to tissue such as skin, cornea and gingiva.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1B-D are phase contrast microscope photomicrographs of living cultures of keratinocytes cultured for 72 hours either in the presence of complete HECK-109 FS medium (B), standard HECK 110 medium (C) or standard HECK 109 medium (D).

Figure 2A is a photograph of living cultures of keratinocytes that have been cultured for 1 hour and stained with crystal violate (0.2%). Figures 2B-D are photographs of the results of the same experiment in which the cultures were fixed at 72 hours after the above treatments and stained with crystal violate (0.2%).

Figure 3A is a photomicrograph of a phase contrast microscope image of a living epidermal sheet produced by monolayer culture of keratinocytes in the inventive protein-free defined HECK-110 medium, and refed upon reaching confleuncy with standard HECK medium containing 10% FBS and 1mM Ca^{2+} ions. Figure 3B is a photomicrograph of a living sheet of epidermis released from the plastic substrate of the culture dish by Dispase proteolytic.

Figures 4A-B are photomicrographs of keratinocyte cultures cultured in complete HECK-109 FS medium and treated in an identical manner with 10% FBS and 1mM Ca^{2+} ions to yield a stratified epidermal epithelium.

DETAILED DESCRIPTION OF THE INVENTION

The epithelial cells that can be advantageously cultured with the media of this invention include adult epidermal keratinocytes, adult corneal epithelial cells, ureteral epithelial cells, gingival keratinocytes, fetal epithelial cells and the like. As discussed in the Background section, the cell culture media according to the invention are beneficial in growing any epithelium such as the cornea of the eye and linings of the respiratory, digestive and genitourinary tissues.

The present invention also relates to a method of culturing animal epithelia comprising culturing said cells in the serum-free media disclosed above.

The important aspects of the inventive media include the low levels of HEPES, modified levels of amino acids and a particular range of osmolalities. The acceptable osmolarities of the media of this invention can range from 275 to 310 milliosmols per liter of solution (mosmols). Osmolarity is the concentration of an osmotic solution when measured in osmols or milliosmols per liter of solution. The inventor has also found that the reduced levels of HEPES, the sodium chloride concentration, (which is directly related to osmolarity) and the concentration of the six (6) amino acids, allows for the omission of serum and any foreign protein factor in the medium and is the basis for the basal medium hereinafter designated HECK-109. Further, an additional point of novelty resides in the use of retinyl acetate at a concentration of 0.3-33 ng/ml, which eliminates

the need for protein growth factors EGF or IGF-1. Additional points of novelty relate to the calcium^{2+} ion concentration of 0.7-3.0 mM and the inclusion of β-transforming growth factor at a concentration of 3.0-30 ng/ml for the differentiation medium hereinafter designated HECK-110 DM. An additional inventive media comprises linoleic acid at a concentration of 1-15 ng per ml for cornification of the reformed tissue hereinafter designated HECK-110 CM.

Admittedly, the prior art is replete with numerous cell culture media. For example, the previously discussed Wolfe, Boyce, Wilke and MCDB media are well known and commercially available. However, none of these references have suggested the removal of all protein growth factors and their replacement with retinyl acetate nor disclosed the improvements the inventor has discovered herein. Those improvements relate to the reduced level of HEPES in combination with the specific and specified levels of six very critical amino acids. Further, while many of the references recite that they are serum-free, they are in fact not tissue-extract free as the various prior art media are taught to utilize various tissue extracts such as bovine pituitary extract.

Most of the major nutrients and other factors essential for cell growth are known and have been used previously and in many permutations. However, the concentrations of certain specific components have been newly formulated for the media of this invention. The components have not merely been optimized but rather a significant discovery has been made in that the components of HEPES and the amino acids are an interrelated set of factors and enhancers for the growth of human epithelial cells. The inventor herein has also discovered that this interrelationship of the various components can also avoid the use of feeder layers such as mouse cells, which are used to produce

serum-like growth factors. The novel media of the invention also allows for the avoidance of bovine pituitary extract as taught and suggested by the prior art. As will be demonstrated below, these changes to the media have a profound effect on the media's ability to allow for prolific cell growth and the ultimate differentiation of the cells into a complete epithelium.

An additional aspect of the present invention resides in the discovery that the prior art levels of the aromatic amino acids (histidine, phenylalanine, tryptophan and tyrosine) presented the cultured cells with a rate limiting amount of these vital nutrients. As such, the present inventor has included in his media significantly different amounts of each of these amino acids. Also critically important is the ratio of the amino acids to each other as the ratios impart the ability of this medium to allow the cultured cells to thrive and form a confluent sheet of tissue without the need for serum components or components derived from serum.

The nutrient basal medium designated HECK-109 has as critical components: (i) N-(2-OH-ethyl-)piperazine-N'-(2-ethane sulfonic acid) (hereinafter "HEPES") at 14-22 mM; ii) NaCl at 100-120 mM; and (iii) six (6) key amino acids at about the following concentrations: histidine at 1.0-2.5 x 10^{-4} M; isoleucine at 0.5-5.0 x 10^{-4} M; methionine at 1.0-5.0 x 10^{-4} M; phenylalanine at 1.0-5.0 x 10^{-4} M; tryptophan at 0.5-5.0 x 10^{-4} M; and tyrosine at 10-5.0 x 10^{-4} M. Taken together, HEPES, NaCl and the six (6) key amino acids are superior to any previous media or similar design, in toxicity, osmolarity and support of clonal growth of basal epithelial cells. All media of this invention, except for the CCS, have an osmolarity of between 275 to 310 mosmols.

The novel protein-free defined medium is a serum-free medium for the growth of undifferentiated basal keratinocyte and is based on HECK 109 basal medium and is herein designated HECK-110. HECK-110 consists of HECK 109 supplemented with retinyl acetate at 0.3 – 33 ng/ml. This medium is selective for the growth of normal human keratinocytes, and is essential for the formation of a hole-free monolayer (intact sheet) of undifferentiated epidermal cells, while suppressing growth-arrest and commitment to terminal cell differentiation and the loss of clonogenic protential.

The following Examples are intended to be illustrative and not limitative. Values presented in parenthesis are an acceptable range for the given element, unless stated otherwise.

EXAMPLE 1

Primary and Secondary Culture of Normal Human Epidermal Keratinocytes in HECK-110 Protein-Free Defined Medium

Isolation of Basal Cells and Primary Cultures

Primary cultures of normal human basal epidermal keratinocytes were started by subjecting full-thickness skin samples to enzymatic digestion. Skin obtained from biopsies or autopsies was first cleaned of adhering subdermal fat and the dermis was reduced to less than 3 mm in thickness. The skin sample was then cut into 8 to 12 small pieces (usually 0.5 cm^2). These pieces were floated on top of sterile CCS (Cell Competency Solution). CCS consisted of glucose, 10 mM; KCl, 3 mM; NaCl, 130 mM; $Na_2HPO_4.7H_2O$, 1 mM; phenol red, 3.3 μM; HEPES at 23 mM; (See Shipley, G. D. and Ham, R. G., In Vitro 17:656-670 (1981)) and 0.17% trypsin (w/v) and 100 units/ml of both penicillin and streptomycin. After 14 to 16 hours of digestion at 4° C., the dermis

was separated from the epidermis by a split-dermis technique. This was accomplished by placing the cornified side of the epidermis on a clean sterile polystyrene surface whereupon the epidermis spontaneously detaches, and the dermis is removed with sterile forceps. Trypsin digestion cleaves the skin along a fracture line which separates some of the basal cells with the dermis, but frees other basal cells lying between the dermis and the fracture line just above the basal cell layer.

The trypsin-treated epidermis, so split from the dermis, was enriched for a subpopulation of loosely-associated, clonally competent basal cells. In a series of experiments, the inventor herein discovered that these loosely-associated basal cells are larger than the basal cells that remain associated with the dermis. Moreover, these larger basal cells are separable by cell sorting procedures using a fluorescence-activated cell sorting device. They also have a greater colony-forming ability than the dermis-associated basal cells, as demonstrated by clonal growth experimentation.

The loosely-associated basal cells were collected in ice-cold (0°-4° C.) CCS containing 0.1-1.0% w/v SOTI solution in place of the trypsin. The cell suspension was then filtered on ice through a 100 micrometer sized Nylon mesh using sterile procedures. Filtration removes the cell aggregates and ensures preparation of a single cell suspension. The cells were pelleted by low speed centrifugation (800 x g (gravity), 5 mins.) at 4° C. The CCS containing SOTI was aspirated off and the remaining cells were resuspended by gentle pipetting in CCS, and washed once with ice-cold HECK-109 (serum-free basal nutrient medium; see Example 2 for detailed preparation of this medium). The centrifugation step was repeated as above, and the resulting cell pellet was resuspended in 1 to 2 ml of HECK-109. Cell counts were obtained by standard cell chamber counting

methods. Primary cultures were initiated into HECK-109 FS supplemented with 0.1 (0.05-0.20) mM ethanolamine; 0.1 (0.5-0.20) mM phosphoethanolamine; 0.5 (0.1-1.0) μM hydrocortisone; and 5 μg/ml EGF. Antibiotics which were added at this time can be removed 2 to 3 days later when the proliferating cell cultures are refed fresh HECK-109 FS. The two protein growth factors (EGF and IGF-1) were added aseptically to the medium. All media was sterilized through a commercially available membrane filter (0.2 microns). The initial seeding density for initiating the primary culture is $5x10^3$ basal cells per cm^2 tissue culture flask. Two flasks were set up from an initial yield of 1 to $2x10^6$ cells isolated from the 2 cm^2 piece of skin. It should be appreciated that the same isolation procedure used for basal keratinocytes from skin can be used to obtain other basal epithelia cells from tissues such as cornea, gingiva, ureter and the like.

Secondary Culture Procedure--Secondary cultures may be initiated from either primary cultures or early passage secondary cultures. Early passage secondary cultures were passaged by enzymatic dissociation of cells. This serial passage technique is not standard. It involves the use of ice-cold 0.02% (0.02-0.20) SOTI (w/v) in CCS as detailed above for initiating primary cultures. Secondary cultures were seeded at an initial cell density of 1000 cells per cm^2 and re-fed HECK-110 medium.

The procedure for calculating colony forming efficiency (CFE) of the basal cells recovered from the epidermis and used to initiate a primary culture is based upon setting up duplicate primary cultures at 5000 cells per cm^2 as described above, and then to count the number of cells which attach and later form a colony of at least 8 or more cells, three days after seeding the primary culture. By this method, the percent attachment of epidermal cells was 50 to 60 percent of the input cells.

EXAMPLE 2

Preparation of HECK-110 Basal Nutrient Medium

One aspect of the present invention relates to the preparation of a new media suitable for the large scale amplification of both primary and secondary cultures of normal human epithelial cells, such as keratinocytes, and for conversion of proliferating normal human epithelial monolayer cultures to a fully differentiated tissue transplantable to a human being. More particularly, this Example 2 is directed to the materials and procedures for preparation of a basal nutrient medium (Human Epidermal Cell Keratinocyte, HECK-110), and experiments evidencing its superiority in stimulating epithelial cell growth. The media according to this invention are novel and unobvious by design of the osmolarity, toxicity and pH-buffering properties.

Table 1 below details the concentration of components in basal medium, HECK-110. All biochemicals, growth factors and hormones were purchased from Sigma Chemical Company (St. Louis, Mo., U.S.A.), and all inorganic chemicals were from Fisher Scientific (Pittsburgh, Pa., U.S.A.). All trace elements in Stock T were from Aesor (Johnson Matthey, Inc., Seabrook, N.H., U.S.A., Purotronic Grade). EGF was prepared according to the procedure of Savage, R. C. and Cohen, S. (J. Biol. Chem. 247:7609-7611 (1972)), or purchased from Collaborative Research, Inc., Waltham, Mass.

One liter of HECK-110 was prepared in a separate stock solution fashion as described in Table 1 with respect to Stocks 2 through 10. Medium HECK-110 differs from all other media in the part art by its Stock 1 amino acids, its concentration of NaCl (113 mM; Range 90-140) and HEPES (20 mM; Range 14-22). The concentration of the six (6) amino acids is critical and must be within the following ranges: isoleucine at 0.5-

5.0 x 10^{-4} M; histidine at 0.5-2.5 x 10^{-4} M; methionine at 1.0-5.0 x 10^{-4} M; phenylalanine at 1.0-5.0 x 10^{-4} M; tryptophan at 0.5-5.0 x 10^{-4} M; tyrosine at 1.0-5.0 x 10^{-4} M.

TABLE 1

Composition of Basal Nutrient Medium HECK-110

		Concentration in Final Medium	
Stock	Component	mg/l	mol/l*
1	Arginine.HCl	421.4	2.00×10^{-3}
	Histidine.HCl.H_2O	36.1	1.70×10^{-4}
	Isoleucine allo-free	33.0	1.50×10^{-4}
	Leucine	132.0	1.00×10^{-3}
	Lysine.HCl	36.6	2.00×10^{-4}
	Methionine	45.0	3.0×10^{-4}
	Phenylalanine	50.0	3.0×10^{-4}
	Threonine	23.8	2.00×10^{-4}
	Tryptophan	40.8	2.00×10^{-4}
	Tyrosine	54.0	3.0×10^{-4}
	Valine	70.2	6.00×10^{-4}
	Choline Chloride	27.9	2.00×10^{-4}
	Serine	126.1	1.20×10^{-3}
2	Biotin	0.0146	6.00×10^{-8}
	Calcium Pantothenate	0.285	1.00×10^{-6}
	Niacinamide	0.03363	3.00×10^{-7}
	Pyridoxal·HCl	0.06171	3.00×10^{-7}
	Thiamine·HCl	0.3373	1.00×10^{-6}
	Potassium Chloride	111.83	1.50×10^{-3}
3	Folic Acid	0.79	1.80×10^{-6}
	$Na_2HPO_4 \cdot 7H_2O$	536.2	2.00×10^{-3}
4a	Calcium chloride·$2H_2O$	14.7	1.00×10^{-4}
4b	Magnesium chloride·$6H_2O$	122.0	6.00×10^{-4}
4c	Ferrous sulfate·$7H_2O$	1.39	5.00×10^{-6}
5	Phenol red	1.242	3.30×10^{-6}
6a	Glutamine	877.2	6.00×10^{-3}
6b	Sodium pyruvate	55.0	5.00×10^{-4}
6c	Riboflavin	0.03764	1.00×10^{-7}
7	Cysteine.HCl	42.04	2.40×10^{-4}
8	Asparagine	13.2	1.00×10^{-4}
	Proline	34.53	3.0×10^{-4}
	Putrescine	0.1611	1.00×10^{-6}
	Vitamin B_{12}	0.407	3.00×10^{-7}
	Retinyl Acetate	0.003	3.00×10^{-8}

9	Alanine	8.91	1.00×10^{-4}
	Aspartic Acid	3.99	3.00×10^{-5}
	Glutamic Acid	14.71	1.00×10^{-4}
	Glycine	7.51	1.00×10^{-4}
10	Adenine	12.16	9.00×10^{-5}
	Inositol	18.02	1.00×10^{-4}
	Lipoic Acid	0.2063	1.00×10^{-6}
	Thymidine	0.7266	2.00×10^{-6}
Trace Element T	Copper sulfate	0.0025	1.00×10^{-8}
	Selenic Acid	0.00687	3.00×10^{-8}
	Manganese Sulfate·$5H_2O$	0.000241	1.00×10^{-9}
	Sodium Silicate·$9H_2O$	0.001421	1.00×10^{-7}
	Ammonium Molybdate·$4H_2O$	0.00124	1.00×10^{-9}
	Ammonium Vanadate	0.00059	1.00×10^{-9}
	Nickel Chloride·$6H_2O$	0.00012	5.00×10^{-9}
	Stannous Chloride	0.000113	5.00×10^{-10}
	Zinc Chloride·$7H_2O$	0.1438	5.00×10^{-7}
Solids S	Glucose	1081.0	6.00×10^{-3}
	Sodium Acetate·$3H_2O$	500.0	3.70×10^{-3}
	Sodium Bicarbonate	1176.0	1.40×10^{-2}
	Sodium Chloride	6600.0	1.13×10^{-2}
	HEPES	4700.0	2.00×10^{-2}

*All above components come together to a final volume of 1 liter of distilled and 0.22 μm filtered water.

The concentrations of these six (6) important amino acids have been shown by the inventor to be necessary for sustained basal cell proliferation. By further experimentation, the inventor discovered that superior growth occurs when the osmolarity of the media are between 275 and 310 milliosmoles (mosmols). The osmolarity of the inventive media are critical to proper cell growth.

Through an extensive series of clonal growth experiments in which the osmolarity was held constant at 300 mosmols and the concentration of HEPES varied between 14 to 28 mM, it was also discovered that the inventive media must incorporate HEPES at between 14-22 mM, preferably between 18 and 22 mM with 22 mM being the most

preferred. This is also critical to the media of this invention. Table 2 presents results of clonal growth experiments showing that the design of HECK-109 supports a higher growth rate and a higher colony forming efficiency than a standard MCDB 153 commercial medium.

A most significant aspect of the present invention is that the concentration of 14 to 22 mM concentration HEPES in HECK-109 medium results in a 2 to 3 fold higher colony forming efficiency than that previously attainable. The second significant discovery is that an osmolarity of 280-310 mosmols, most preferably 300 mosmols, of the media permits attainment of higher saturation densities at confluence of the monolayer culture. The third significant discovery is that it is necessary to provide the indicated concentrations of 6 key amino acids present in Stock 1 (typically 2 to 5 times higher concentration than that in commercially available in MCDB 153 medium). This allows human epithelial cell cultures to routinely achieve a cell density equal to or greater than 100,000 cells per cm^2. HECK-109 incorporates these three discoveries in such a way that the media will allow for and fully support the formation of a complete reformed human epithelium.

EXAMPLE 3

Growth of Secondary Cultures of Normal Human Epidermal Keratincytes in HECK-110 Protein-free Defined Medium

Human keratinocyte cultures were initiated from neonatal foreskin as described in Example 1, and then placed in secondary culture in complete HECK-109 FS medium. The purpose of the following experiment was to determine the effect of retinyl acetate on

the proliferation of keratinocyte cultures refed HECK-109 basal medium lacking EGF and IGF-1 and supplemented with hydrocortisone, ethanolamine, and phosphoethanolamine. For this purpose, duplicate secondary cultures were refed either 1) complete HECK-109 FS medium (positive control) containing 0.1 mM Ca^{2+}, 2) standard HECK-109 medium, i.e., basal medium supplemented with only hydrocortisone, ethanolamine, and phosphoethanolamine, and containing 0.1 mM Ca^{2+} and 3) standard HECK-109 supplemented with retinyl acetate (3 x 10^{-8}M) (now called HECK-110). Figures 1B-D are phase contrast microscope photomicrographs of living cultures of keratinocytes cultured for 72 hours either in the presence of complete HECK-109 FS medium (B), standard HECK 110 medium (C) or standard HECK 109 medium (D). Two important observations were made. First, the colony morphology of retinyl acetate treated keratinocyte cultures displays a loose colony configuration, which is characteristic of proliferating cultures and the like that observed for the cultures maintained in growth factor replete medium. In addition, many dividing cells were observed both in the growth factor supplemented and in cultures refed growth factor-deficient medium supplemented with retinyl acetate. None were observed in cultures refed growth factor-deficient medium. The latter displayed a compact colony morphology characteristic of growth-arrested keratinocytes that have committed to terminal cell differentiation. This experiment has been repeated four times with the same results.

Figure 2A is a photograph of living cultures of keratinocytes that have been cultured for 1 hour and stained with crystal violate (0.2%). Figures 2B-D are photographs of the results of the same experiment in which the cultures were fixed at 72

hours after the above treatments and stained with crystal violate (0.2%). The photographs show that cultures fed complete growth factor containing medium (B) had the most colonies, while cultures refed growth factor-deficient medium supplemented with retinyl acetate (D) has many more colonies than cultures refed only growth-factor deficient standard medium (C).

EXAMPLE 4

Formation of Living Epidermal Sheet Promoted by Protein-Free Medium HECK-110

For this example, a viable living epidermal sheet of tissue of normal human keratinocytes was produced by first isolating keratinocytes by use of cell competency solution (CCS, U.S. Patent No. 5,795,281 to J. Wille), and then culturing the primary culture in complete keratinocyte growth medium (HECK-109 FS), as described in U.S. Patent No. 5,686,307 to J. Wille. Early passage cultures were further sub-cultivated in complete HECK-109 FS. Duplicate low cell density ($5x10^3/cm^2$) culture refed protein-free defined HECK-110 medium for an additional 5 days until a confluent monolayer was formed. The cultures were then washed twice with HECK-109 standard media, i.e., basal medium containing ethanolamine (10^{-4} M, and phophoethanolamine (10^{-4} M), and hydrocortisone ($5x\ 10^{-7}$ M), and refed HECK-110 standard medium containing 10% fetal bovine serum and 1mM Ca^{2+} ions for an additional two days. Figure 3A is a photomicrograph of a phase contrast microscope image of a living epidermal sheet produced by monolayer culture of keratinocytes in the inventive protein-free defined HECK-110 medium, and refed upon reaching confleuncy with standard HECK medium containing 10% FBS and 1mM Ca^{2+} ions. Figure 3B is a photomicrograph of a living sheet of epidermis released from the plastic substrate of the culture dish by Dispase

proteolytic enzyme treatment (2mg/ml for 20 minutes). The general appearance of and morphology of the reformed human epidermis so formed was similar to duplicate keratinocyte cultures cultured in complete HECK-109 FS medium and treated in an identical manner with 10% FBS and 1mM Ca^{2+} ions to yield a stratified epidermal epithelium (Figures 4A and 4B).

INDUSTRIAL APPLICABILITY

Significant improvement allows for cell growth without protein or peptide growth factors where retinyl acetate is substituted at physiologically acceptable concentrations. The present invention produces proliferating cultures of keratinocytes that do not require EGF or IGF-1 or any other added protein growth factor. The present invention is directed to the design and formulation of the various novel HECK 110 media, which provide for the differentiation of pluripotent basal epithelial cells to a fully differentiated human epithelium in vitro. HECK-110 is the complete medium for cell growth: HECK-110 DM is for the induction of differentiation and formation of a Malphigian layer and HECK-110 CM is designed for the induction of cellular differentiation in a pre-existing reformed tissue produced by HECK-110 DM. The invention also relates to a method of sequential control for the in vitro construction of a histologically complete living epithelium. The tissue derived from the media and methods of the invention have application for in vitro testing of pharmaceuticals and topical drugs; screening of toxicants, carcinogens, complete or incomplete tumor promoters; evaluation of infective human agents including viruses, e.g., human papilloma viruses, Herpes-simplex viruses and Epstein-Barr virus; and screening of cosmetics.

Most importantly the present invention allows for the use of autologously-derived tissue for transplantation in the treatment of burns or other trauma. Further, the present invention would allow for autologous production of skin, corneal tissue, gingival tissue, ureter tissue and other epithelium for transplant to a patient in need thereof.

Numerous modifications and variations in the invention are expected to occur to those skilled in the art upon considerations of the foregoing descriptions. The invention should not be construed as limited to the preferred embodiments and modes of preparation described herein, since these are to be regarded as illustrative rather than restrictive.

We claim:

1. A protein-free defined medium for culturing epidermal keratinocytes comprising:

a) N- (2-OH-ethyl-)piperazine-N -(2-ethane-sulfonic acid) at a concentration of 14-22 mM;

b) sodium chloride at a concentration of 100-120 mM;

c) histidine at a concentration of 0.1-0.25 mM;

d) isoleucine at a concentration of 0.05-0.5 mM;

e) methionine at a concentration of 0.1-0.5 mM;

f) phenylalanine at a concentration of 0.1-0.5 mM;

g) tryptophan at a concentration of 0.05-0.5 mM;

h) tyrosine at a concentration of 0.1-0.5 mM; and

i) retinyl acetate at a concentration of 0.3-33 ng/ml.

2. A protein-free defined medium for culturing epidermal keratinocytes comprising:

a) N- (2-OH-ethyl-)piperazine-N'-(2-ethane-sulfonic acid) at a concentration of 14-22 mM;

b) sodium chloride at a concentration of 100-120 mM;

c) calcium^{2+} ions at a concentration of 0.7-3.0 mM;

d) histidine at a concentration of 0.1-0.25 mM;

e) isoleucine at a concentration of 0.05-0.5 mM;

f) methionine at a concentration of 0.1-0.5 mM;

g) phenylalanine at a concentration of 0.1-0.5 mM;

h) tryptophan at a concentration of 0.05-0.5 mM;

i) tyrosine at a concentration of 0.1-0.5 mM;

j) β-transforming growth factor at a concentration of 3.0-30 ng/ml; and

k) retinyl acetate at a concentration of 0.3-33 ng/ml.

3. A protein-free defined medium for culturing epidermal keratinocytes comprising:

a) N-(2-OH-ethyl-)piperazine-N'-(2-ethane-sulfonic acid) at a concentration of 14-22 mM;

b) sodium chloride at a concentration of 100-120 mM;

c) calcium.sup.2+ ions at a concentration of 0.7-3.0 mM;

d) histidine at a concentration of 0.1-0.25 mM;

e) isoleucine at a concentration of 0.05-0.5 mM;

f) methionine at a concentration of 0.1-0.5 mM;

g) phenylalanine at a concentration of 0.1-0.5 mM;

h) tryptophan at a concentration of 0.05-0.5 mM;

i) tyrosine at a concentration of 0.1-0.5 mM;

j) linoleic acid at a concentration of 1-15 ng/ml; and

k) retinyl acetate at a concentration of 0.3-33ng/ml.

4. A method for the formation of a histologically complete stratified epidermis comprising the steps of:

a) isolation of basal stem cells from animal epithelium using a solution comprising:

i) glucose at a concentration of about 10 mM;

ii) N-(2-OH-ethyl-)piperazine-N'-(2-ethanesulfonic acid) at a concentration of 16-22 mM;

iii) sodium chloride at a concentration of 90-140 mM;

iv) potassium chloride at a concentration of about 3 mM;

v) sodium orthophosphate (Na.sub.2 HPO.sub.4.7H.sub.2 O) at a concentration of 1 mM;

vi) phenol red at a concentration of 0.0033 mM;

vii) about 100 units of penicillin per ml of solution;

viii) about 100 units of streptomycin per ml of solution; and

ix) trypsin at a concentration of 0.1%-0.2% w/v;

b) recovering said isolated basal stem cells using a solution comprising:

i) glucose at a concentration of about 10 mM;

ii) N-(2-OH-ethyl-)piperazine-N'-(2-ethanesulfonic acid) at a concentration of 16- 22 mM;

iii) sodium chloride at a concentration of 90-140 mM;

iv) potassium chloride at a concentration of about 3 mM;

v) sodium orthophosphate (Na.sub.2 HPO.sub.4.7H.sub.2 O) at a concentration of 1 mM;

vi) phenol red at a concentration of 0.0033 mM;

vii) about 100 units of penicillin per ml of solution;

viii) about 100 units of streptomycin per ml of solution; and

ix) soy bean trypsin inhibitor at a concentration of 0.1%-1.0% w/v;

c) culturing said isolated basal stem cells in a medium to form a confluent sheet of undifferentiated epithelial tissue, said medium comprising:

i) N-(2-OH-ethyl-)piperazine-N'-(2-ethanesulfonic acid) at a concentration of 14-22 mM;

ii) sodium chloride at a concentration of 100-120 mM;

iii) histidine at a concentration of 0.1-0.25 mM;

iv) isoleucine at a concentration of 0.05-0.5 mM;

v) methionine at a concentration of 0.1-0.5 mM;

vi) phenylalanine at a concentration of 0.1-0.5 mM;

vii) tryptophan at a concentration of 0.05-0.5 mM;

viii) tyrosine at a concentration of 0.1-0.5 mM; and

ix) retinyl acetate at a concentration of 0.3-33 ng/ml;

d) culturing said sheet of undifferentiated epithelial tissue in a differentiation medium to form a sheet of differentiated and stratified tissue, said differentiation medium comprising:

i) N-(2-OH-ethyl-)piperazine-N'-(2-ethanesulfonic acid) at a concentration of 14-22 mM;

ii) sodium chloride at a concentration of 100-120 mM;

iii) calcium^{2+} ions at a concentration of 0.7-3.0 mM;

iv) histidine at a concentration of 0.1-0.25 mM;

v) isoleucine at a concentration of 0.1-05 mM;

vi) methionine at a concentration of 0.1-0.5 mM;

vii) phenylalanine at a concentration of 0.1-0.5 mM;

viii) tryptophan at a concentration of 0.05-0.5 mM;

ix) tyrosine at a concentration of 0.1-0.5 mM;

x) β-transforming growth factor at a concentration of 3.0-30 ng/ml; and

xi) retinyl acetate at a concentration of 0.3-33 ng/ml; and

e) culturing said differentiated and stratified tissue in a cornification medium to form a cornified epithelium, said cornification medium comprising:

i) N-(2-OH-ethyl-)piperazine-N'-(2-ethanesulfonic acid) at a concentration of 14- 22 mM;

ii) sodium chloride at a concentration of 100-120 mM;

iii) calcium.sup.2+ ions at a concentration of 0.7-3.0 mM;

iv) histidine at a concentration of 0.1-0.25 mM;

v) isoleucine at a concentration of 0.05-0.5 mM;

vi) methionine at a concentration of 0.1-0.5 mM;

vii) phenylalanine at a concentration of 0.1-0.5 mM;

viii) tryptophan at a concentration of 0.05-0.5 mM;

ix) tyrosine at a concentration of 0.1-0.5 mM;

x) linoleic acid at a concentration of 1-15 ng/ml;

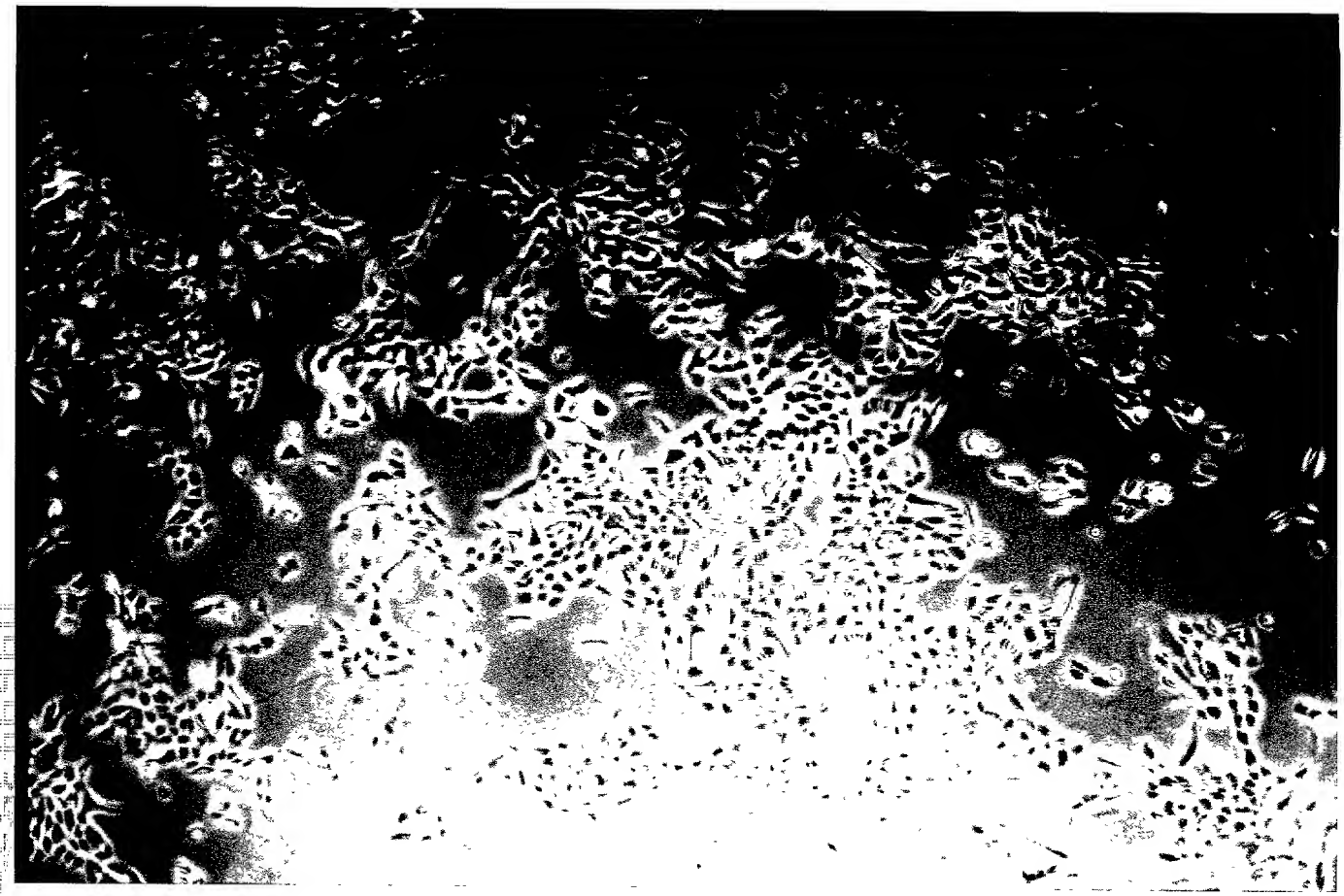
xi) retinyl acetate at a concentration of 0.3-33 ng/ml.

5. The method according to claim 4 wherein said histologically complete epidermis is human epidermis.

6. The method according to claim 4, further comprising the step of using serum or tissue extract or animal derived factors or other xenobiotics in combination with Ca^{2+} ions (1-2 mM) in order to form a stratified keratinizing epithelium.

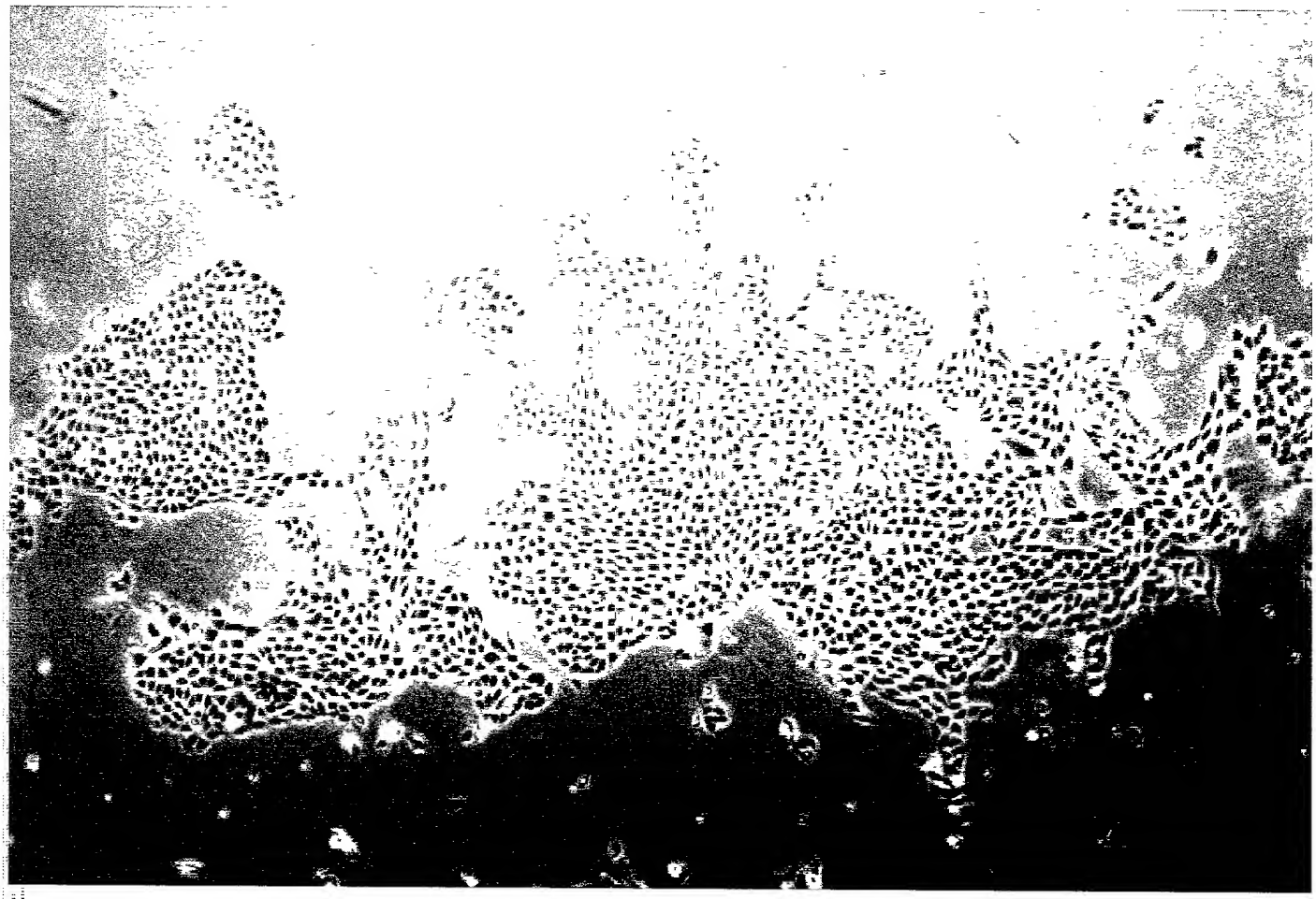
ABSTRACT

Improvements are made to a novel media that replace the requirement for all protein growth factors by the addition to the medium of physiological concentrations of retinyl acetate. The media are serum-free, companion cell or feeder layer-free and organotypic, matrix free solutions for the cultivation of clonally competent basal keratinocytes. The media and methods are useful in the production of epidermal epithelial tissue that is suitable for skin grafting.



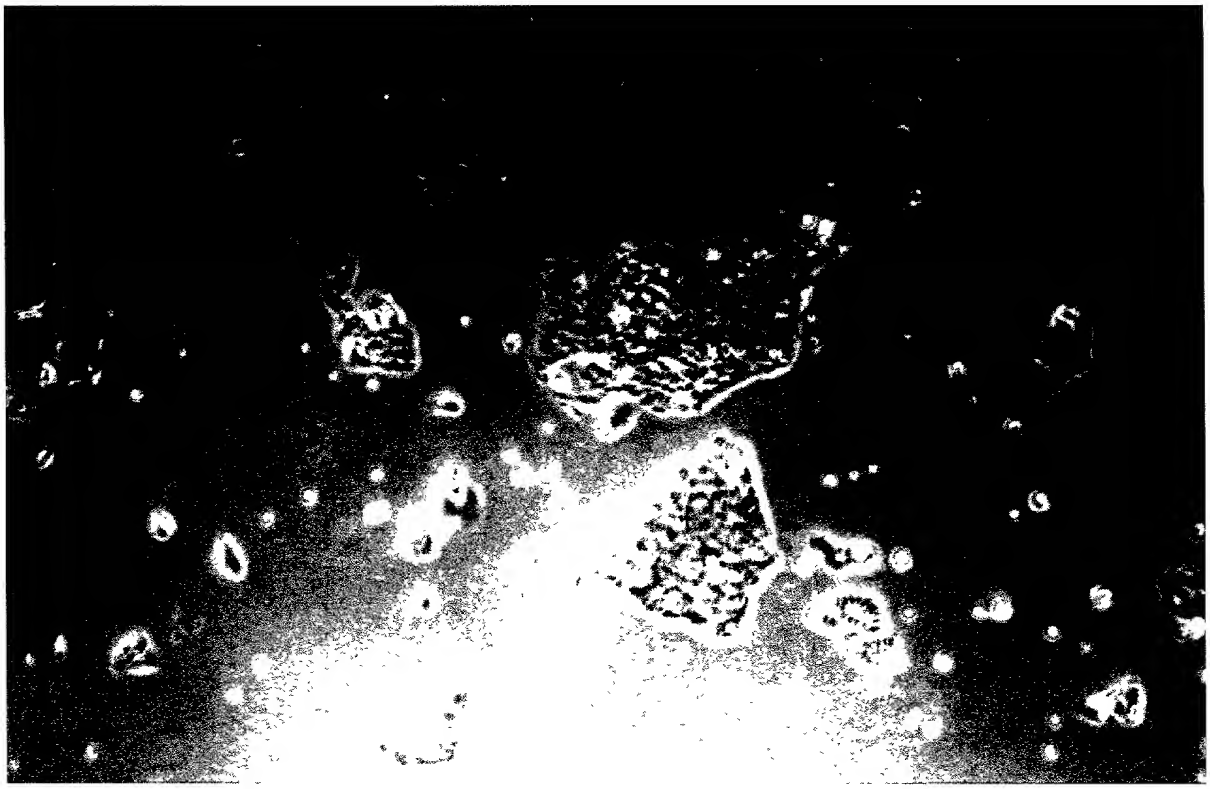
EXPERIMENT 2 - PLATE B - 72 HRS
COMPLETE MEDIA W/ PROTEIN GROWTH FACTORS
LIVE CELLS - PHASE CONTRAST MICROSCOPY - 160X

FIG. 1B



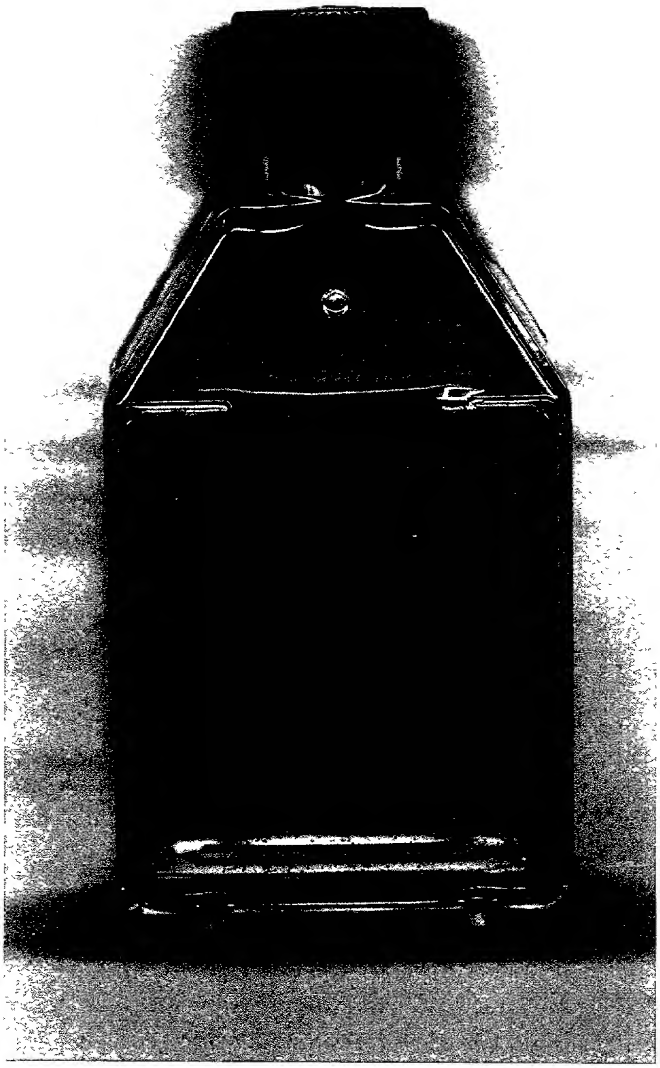
EXPERIMENT 2 - PLATE C - 72 HRS
STANDARD MEDIA W/ RETINYL ACETATE
NO PROTEIN GROWTH FACTORS
LIVE CELLS - PHASE CONTRAST MICROSCOPY - 160X

FIG. 1C



EXPERIMENT 2 - PLATE D - 72 HRS
STANDARD MEDIA W/ NO RETINYL ACETATE &
NO PROTEIN GROWTH FACTORS
LIVE CELLS - PHASE CONTRAST MICROSCOPY - 160X

FIG. 1D

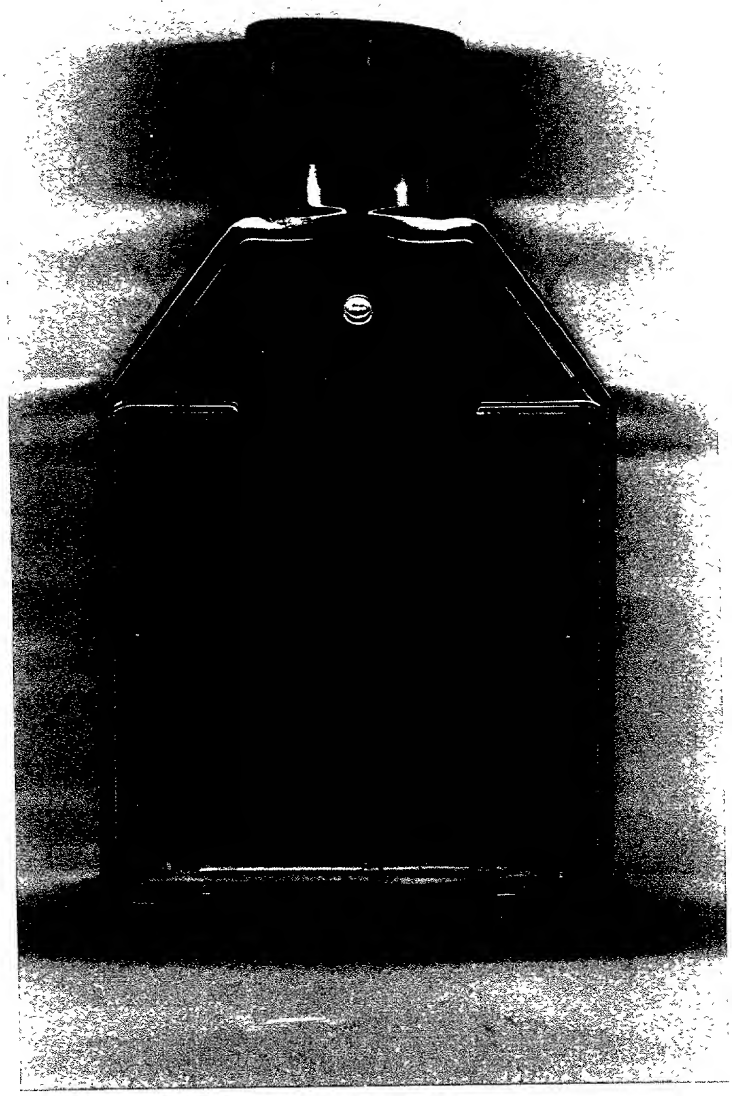


EXPERIMENT 2 - PLATE A - 1 HR
TOTAL CELL PLATING
CELLS FIXED AND STAINED -1.25X ACTUAL SIZE
RELATIVE OPTICAL DENSITY UNITS = *1.0*

FIG. 2A

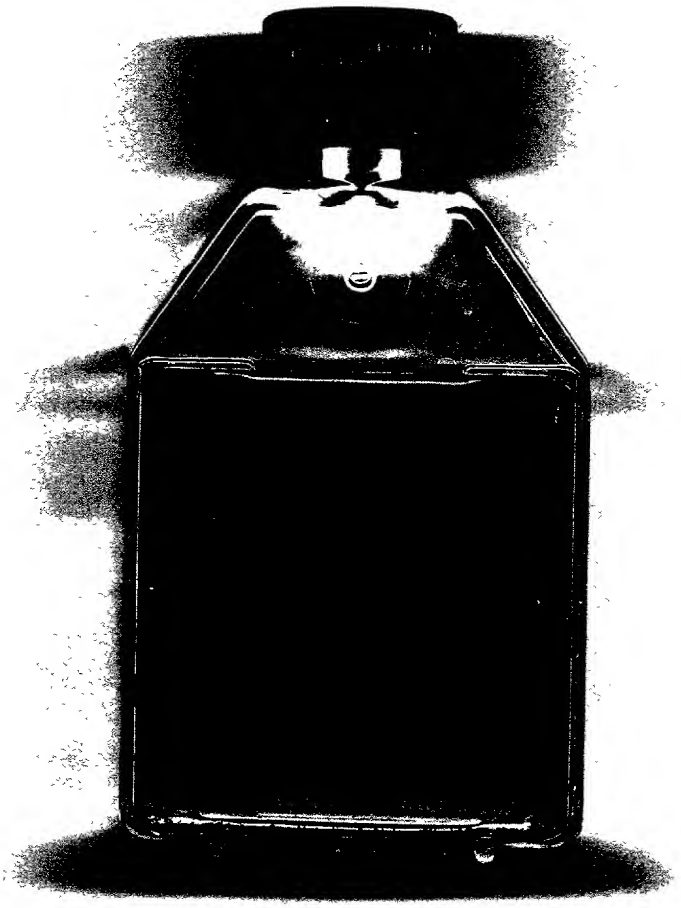
EXPERIMENT 2 - PLATE B - 72 HRS
COMPLETE MEDIA W/ PROTEIN GROWTH FACTORS
CELLS FIXED AND STAINED -1.25X ACTUAL SIZE
RELATIVE OPTICAL DENSITY UNITS TO PLATE A = *33.8*

FIG. 2B



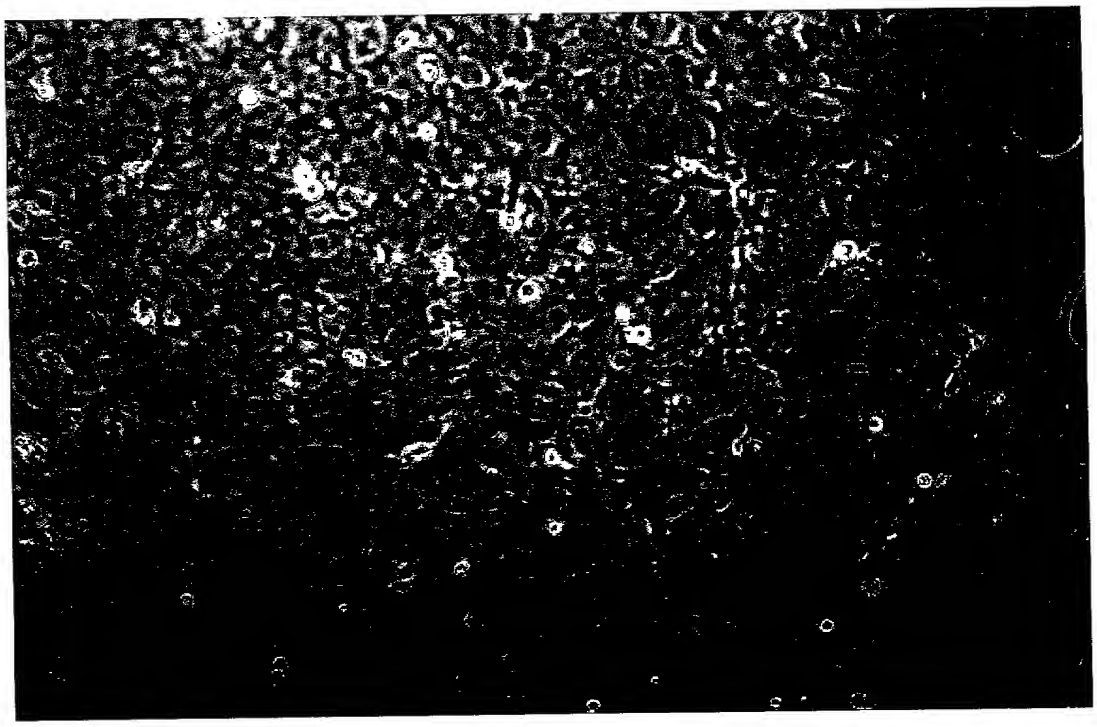
EXPERIMENT 2 - PLATE C - 72 HRS
STANDARD MEDIA W/ RETINYL ACETATE
NO PROTEIN GROWTH FACTORS
CELLS FIXED AND STAINED -1.25X ACTUAL SIZE
RELATIVE OPTICAL DENSITY UNITS TO PLATE A = *29.7*

FIG. 2C



EXPERIMENT 2 - PLATE D - 72 HRS
STANDARD MEDIA W/ NO RETINYL ACETATE &
NO PROTEIN GROWTH FACTORS
CELLS FIXED AND STAINED -1.25X ACTUAL SIZE
RELATIVE OPTICAL DENSITY TO PLATE A = *6.7*

FIG. 2D

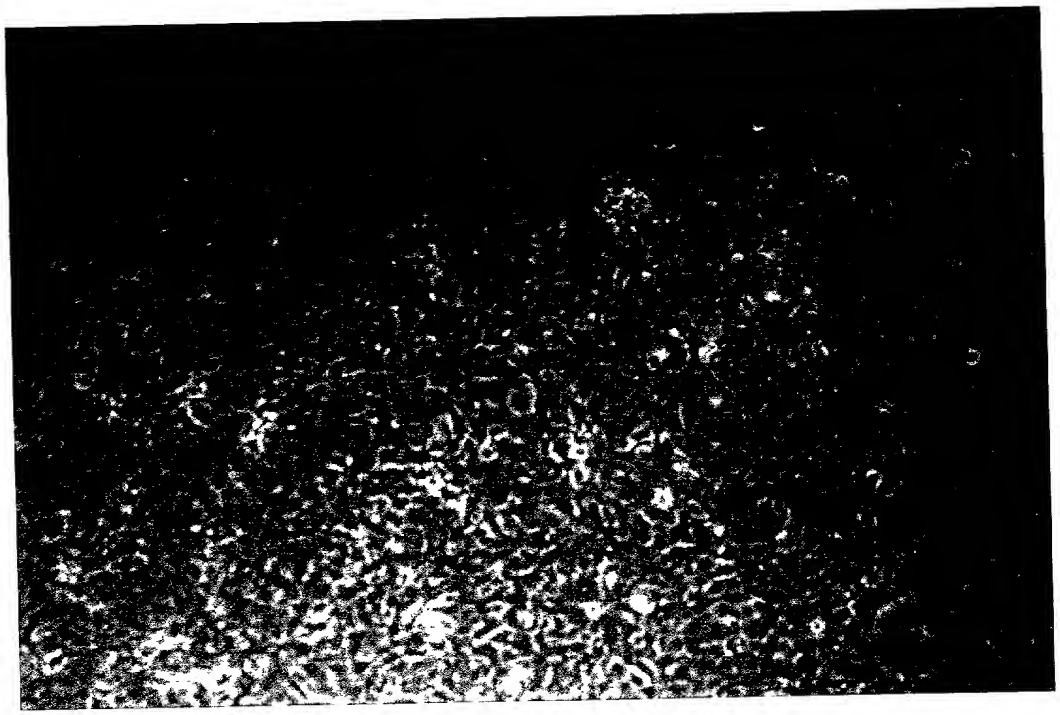


RETINYL ACETATE CULTURE TREATMENT
PRE-DISPASE RELEASE OF SKIN SHEET
PHASE CONTRAST MICROSCOPE
20X MAGNIFICATION

FIG. 3A

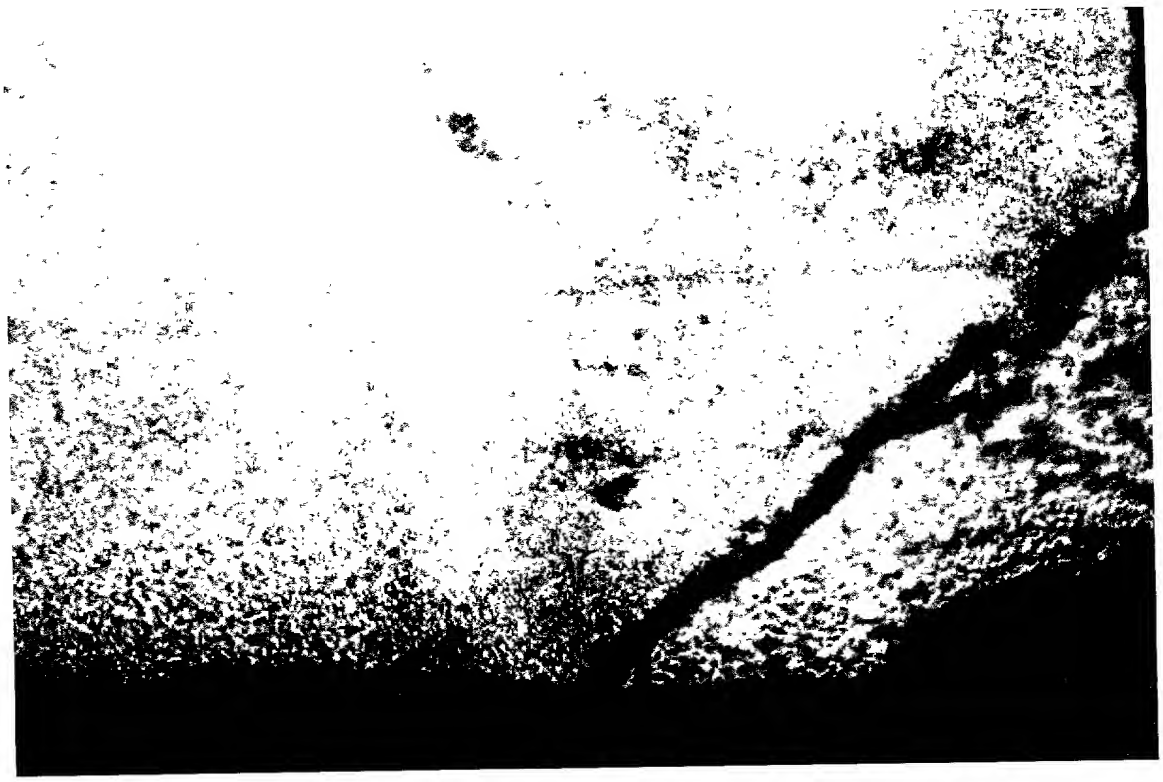
RETINYL ACETATE
WITHOUT PROTEIN GROWTH FACTORS
***RETINYL ACETATE* - SKIN SHEET**
TREATED W/ FBS +1mm Ca^{++}
DISPASE TOTAL RELEASE
PHASE CONTRAST MICROSCOPE
20X MAGNIFICATION

FIG. 3B



TOTAL CELL PLATING
CONTROL
PHASE CONTRAST MICROSCOPE
20X MAGNIFICATION

FIG. 4A



WITH PROTEIN GROWTH FACTORS
TREATED W/ FBS + 1mm Ca^{++}
***CONTROL* - SKIN SHEET DISPASE RELEASED**
PHASE CONTRAST MICROSCOPE
20X MAGNIFICATION

FIG. 4B

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

[X] Declaration Submitted with Initial Filing OR [ ] Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	HYG 1194-011D
First Named Inventor	Wille, Jr.
COMPLETE IF KNOWN	
Application Number	/
Filing Date	Herewith
Group-Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROTEIN-FREE DEFINED MEDIA FOR THE GROWTH OF NORMAL HUMAN KERATINOCYTES

(Title of the Invention)

the specification of which

[X] is attached hereto

OR

[ ] was filed on (MM/DD/YYYY) [ ] as United States Application Number or PCT International Application Number [ ] and was amended on (MM/DD/YYYY) [ ] (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

[ ] Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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DECLARATION – Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 11 2, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
09/271,777	03/18/99	
08/893,195	07/15/97	5,834,312
08/500,744	07/11/95	5,686,307
08/318,221	10/05/94	

☒ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: ☒ Customer Number [ ] → [barcode] OR ☐ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))	Family Name or Surname
John J.	Wille, Jr.

Inventor's Signature	John J. Wille, Jr.					Date	9/23/10
Residence: City	Trenton	State	NJ	Country	USA	Citizenship	USA
Post Office Address	9 Georgetown-Chesterfield Road						
Post Office Address							
City	Trenton	State	NJ	ZIP	08625	Country	USA

☐ Additional inventors are being named on the supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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DECLARATION -- Supplemental Priority Data Sheet

Additional foreign applications:

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐

Additional provisional applications:

Application Number	Filing Date (MM/DD/YYYY)

Additional U.S. applications:

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (If applicable)
08/184,905		01/21/94	
08/063,247		05/18/93	
07/471,976		01/29/90	5,292,655

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